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Phenmedipham–Ozone Pollution Interactions in Sugarbeet (*Beta vulgaris* L. cv. Saxon): A Physiological Study

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Abstract: Actively growing sugarbeet is treated with the post-emergent herbicide phenmedipham at times when ozone pollution episodes are likely to occur. There is a possibility of an interaction occurring between ozone and phenmedipham as both treatments produce similar effects in susceptible plants, such as a reduction in growth and photosynthesis and an increase in the activities of endogenous antioxidant enzymes. To investigate this likelihood, laboratory experiments were conducted in which two- to three-leaf sugarbeet plants (*Beta vulgaris* L. cv. Saxon) were exposed to a simulated two-day ozone episode (100 nl litre^{-1}, 7 h day^{-1}) followed three days later by treatment with field rate phenmedipham (1·14 kg AI ha^{-1}). Growth analysis indicated that an interaction was occurring in which plants treated with ozone and phenmedipham had less reduction in shoot fresh weight than expected. Exposure to phenmedipham alone or ozone followed by phenmedipham reduced net photosynthesis by over 50% and transpiration rate by 30%. The activities of antioxidant enzymes such as catalase, guaiacol peroxidase and superoxide dismutase were stimulated by both treatments individually, but to a greater extent when ozone and phenmedipham were combined. For example, three days after herbicide treatment, the activity of superoxide dismutase increased by 20% in plants treated with ozone alone, 20% in plants treated with phenmedipham alone and 85% in plants that were treated with ozone followed by phenmedipham. We conclude that ozone pollution may predispose sugarbeet to tolerate the herbicide phenmedipham by enhancing the activity of the endogenous antioxidant detoxification enzyme system.

Key words: ozone pollution, phenmedipham, sugarbeet, antioxidant enzymes, photosynthetic inhibitors.

1 INTRODUCTION

Phenmedipham (methyl 3-(3-methylcarbaniloyloxy)carbanilate) is the most important post-emergence herbicide used in sugarbeet in the UK.[1] It controls broadleaf- and some grass-weeds[2–4] by the inhibition of photosynthetic electron transport.[5] However, application of phenmedipham to sugarbeet can result in reductions in root and sugar yields to the crop, depending on the environmental conditions during and after spraying. For example, application during periods of warm and sunny weather has been shown to decrease plant dry weight by 73% compared to 36% when the temperatures were lower (24–52 days after spraying; 26 or 10°C).[6] In an earlier study, Cantwell & Norris[7] showed that increasing temperatures after phenmedipham application resulted in severe injury and immediate cessation of growth of the crop. Since hot, sunny and still conditions are frequently associated with episodes of photochemical ozone pollution,[8] there is a possibility that the reduced crop safety of phenmedipham reported in these two studies could have resulted from a synergistic interaction between the herbicide and ozone. In 1995, most of the sugarbeet in the UK was sown by 8 April.[9] One month later, in early May, ozone pollution episodes in excess of 100 nl litre^{-1} were recorded for

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four consecutive days, leading to the potential for interactions to occur between ozone and the herbicides applied when the crop is at the seedling stage.[10] This possibility has been investigated in a laboratory study of the physiological basis of ozone: phenmedipham interactions.

Several studies have indicated that interactions can occur between herbicides and ozone pollution.[11] The nature of the interaction depends on a number of factors. Firstly, the timing of the spray in relation to an ozone episode. For example, exposure of the weed *Abutilon theophrasti* Medic. (velvetleaf) to ozone prior to chlorsulfuron treatment resulted in an additive interaction, yet when the treatments were reversed, an antagonistic effect was reported.[12] Secondly, crop sensitivity to both the pollutant and the herbicide should be considered. Treatment with chloramben prior to ozone exposure of two cultivars of *Nicotiana tabacum* L. (tobacco) resulted in synergistic and additive effects on Delhi 34 (ozone-tolerant) and White Gold (ozone-sensitive) respectively, indicating an apparent loss of tolerance to ozone by Delhi 34.[13] However, when pretreated with pebulate, the effects were additive and synergistic, respectively. Thirdly, ozone concentration can influence the direction of the interaction. Thus, exposure of *Zea mays* L. to 200 nl litre^{-1} ozone after treatment with atrazine resulted in an additive response, whereas the interaction was antagonistic at 300 nl litre^{-1}.[14] Lastly, environmental conditions at the time of interaction are also important in ozone: herbicide interactions. For example, exposure of *Lycopersicon esculentum* Mill. (tomato) to 300 nl litre^{-1} ozone prior to metribuzin treatment with low light produced an additive interaction,[15] whereas the interaction was antagonistic in high light conditions. These examples indicate the need for carefully defined experiments to investigate the potential for interaction between ozone and a particular herbicide.

There are some similarities in the mechanism of action of ozone and herbicides such as phenmedipham that inhibit photosynthesis, since active oxygen species have been implicated in both cases.[16,17] When ozone enters the leaf via the stomata, it is thought to dissolve rapidly in water and be converted into oxygen species such as superoxide, hydroxyl radicals and hydrogen peroxide at the plasma membrane.[18] The formation of such species prior to symptom appearance has been demonstrated in *Pisum sativum* L. and *Phaseolus vulgaris* L. using electron spin resonance.[19] Similarly, when the light reactions of photosynthesis are inhibited by the binding of a herbicide to the D_1 protein in photosystem II, the resulting excess excitation energy is eventually transferred to oxygen resulting in the generation of singlet oxygen and other toxic species. Regardless of the cause of the formation of active oxygen species, the net effect is membrane, protein and nucleic acid damage.[20,21] However, plants contain several enzymatic and non-enzymatic protective mechanisms to combat these potentially damaging oxygen species including scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT) and non-specific peroxidases (GPX). Damage is also prevented by the actions of ascorbic acid (Vit C), reduced glutathione (GSH), α-tocopherol (Vit E) and carotenoids.[22]

The interaction of ozone pollution and phenmedipham on sugarbeet cv. Saxon is described in this paper. The interaction is quantified and examined in relation to physiological changes in treated plants, including photosynthesis and the total cellular activities of key active-oxygen scavenging enzymes. Particular emphasis is placed on establishing a time-course of changes in the antioxidant defence systems of plants treated with ozone alone, phenmedipham alone and ozone followed by phenmedipham.

2 MATERIALS AND METHODS

2.1 Growth of plants

Sugarbeet (*Beta vulgaris* L. cv. Saxon) was sown in 7-cm diameter pots containing J. Arthur Bowers multipurpose compost. The pots were initially maintained in a glasshouse at 22°C and 14 h daylength (natural light supplemented with sodium halide lamps during the winter). At the young seedling stage (14 days after sowing), the plants were thinned to two per pot, and the pots were transferred to a Fitotron growth cabinet (Sanyo, Loughborough, UK) at 21°C day/10°C night, 50% relative humidity and 180 μmole photons m^{-2} s^{-1} PAR, 14 h daylength. The plants were returned to the growth cabinet after exposure to ozone or filtered air, and after the application of herbicides.

2.2 Ozone exposure

Plants were exposed to ozone (21 days after sowing) in a closed system consisting of four 0·8 × 0·8 × 0·8 m perspex chambers in the same glasshouse compartment. Ambient air was initially drawn through a Purafil and charcoal filter (Jones & Attwood Ltd, Stourbridge, UK) to remove ambient ozone, nitrogen oxides and sulphur dioxide and distributed to all chambers at a flow rate equivalent to one air change every 3 min. The distribution of air within the chambers was aided by 8-cm diameter fans placed perpendicular to the incoming air. Ozone was generated by passing zero grade oxygen around a UV lamp (Light O_3 Clean A/S, Denmark) and added to the filtered air of two of the chambers. The ozone concentration within the chambers was analysed using a Dasibi 1003 PC ozone analyser (Dasibi Environmental Corp., California, USA). Ozone concentrations were calculated using the

30-min means to produce a 7-h mean from 10 am to 5 pm on each day of exposure.

Sugarbeet plants at the two- to three-leaf stage were exposed to either 100 nl litre^{-1} of ozone, or filtered air for 7 h day^{-1} (10 am to 5 pm GMT) on two consecutive days. This exposure regime was chosen as it was representative of a typical ozone episode in the UK.[8] Following exposure, the plants were returned to the growth cabinet.

2.3 Herbicide treatment

Three days after exposure to ozone or filtered air, the plants were treated with phenmedipham 114 g litre^{-1} EC ('Betanal' E; AgrEvo, UK, Ltd) in distilled water, or with distilled water alone. An experimental sprayer was used to apply the herbicide at a rate equivalent to recommended field rate (1·14 kg AI ha^{-1}, 240 litre ha^{-1}; Teejet 80° flat fan nozzle; 300 kPa pressure). Plants not treated with 'Betanal' E were sprayed with distilled water rather than dilutions of a blank formulation to avoid any interaction which might occur between ozone and the formulants in the latter.

2.4 Assessment of symptoms and fresh weight

Seven days after herbicide treatment, visible injury (contact damage, chlorosis, necrosis or epinasty) was assessed as a percentage of leaf area. The plants were excised at root level, fresh weight was recorded, and 1 g of tissue from the first pair of leaves was analysed for pigment content using a modified method of Arnon.[23] Calculations were carried out using the equations for 80% (v/v) aqueous acetone of Lichtenhaler & Wellburn.[24]

2.5 Photosynthesis

An Infrared Gas Analyser (IRGA, LCA4, Analytical Development Company, Hoddesdon, UK) was used to measure gaseous exchange by a leaf enclosed within an ADC portable leaf chamber. An open system was utilised in which ambient air was passed through the chamber on a continuous basis.[25] Photosynthetic rate (μmol CO_2 m^{-2} s^{-1}) and transpiration rate (mol H_2O m^{-2} s^{-1}) were calculated. Daily measurements were made between 1 and 3 pm GMT at 300 μmol photons m^{-2} s^{-1} PAR and 20°C for the first and second leaves of eight plants per treatment per replicate.

2.6 Enzymes

Approximately 1 g of leaf tissue was frozen in liquid nitrogen and ground to a fine powder using a pestle and mortar. This was transferred with a cardboard spatula to a centrifuge tube (50 ml) containing potassium phosphate buffer (4·25 ml; 100 mM, pH 7·0), ascorbate (0·50 ml, 100 mM), dicthylenetriamine penta(acetic acid) (DTPA; 0·25 ml, 100 mM) and polyvinyl polypyrrolidine (PVPP; 0·2 g).[26] An Ultra Turrax was used to homogenise this mixture at high speed for 20 s and the homogenate was centrifuged at 20 000*g* for 10 min at 4°C. The supernatant was desalted through a Sephadex G-25 PD-10 column (Pharmacia) and the protein content of each sample was measured in triplicate using a modified Bradford[27] method. All enzyme activities were expressed on a protein basis.

Superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), glutathione reductase (GTR; EC 1.6.4.2), glutathione-*S*-transferase (GST; EC 2.5.1.18), catalase (CAT; EC 1.11.1.6) and guaiacol peroxidase (GPX; EC 1.11.1.7) were assayed from the same extraction.

All assays were conducted in a reaction volume of 1 ml using an extract volume of 50 μl in each case, whereas a variable reaction volume was used for SOD depending on the activity of the extract. APX, MDHAR and GTR were assayed immediately after extraction to prevent decay of activity.[26]

SOD was assayed according to the competitive inhibitor method of Beyer & Fridovich.[28] APX was assayed by the method of Nakano & Asada[29] using a reaction mixture containing potassium phosphate buffer (100 mM, pH 7·0) with DTPA (0·2 mM), ascorbate (10 mM) and hydrogen peroxide (5 mM). The oxidation of ascorbate by hydrogen peroxide to monodehydroascorbate was followed spectrophotometrically at 290 nm. A modified method of Hossain, Nakano and Asada[30] was used to assay for MDHAR in which the oxidation of NADH was measured as a decrease in absorbance at 340 nm of a reaction mixture containing potassium phosphate buffer (100 mM, pH 7·8) with DTPA (0·2 mM), ascorbate (10 mM), NADH (3 mM) and ascorbate oxidase (0·2 units 50 μl^{-1}).

GTR was measured by the decrease in absorbance at 340 nm due to the oxidation of NADPH, using a reaction mixture containing potassium phosphate buffer (100 mM, pH 7·8) containing DTPA (0·2 mM), oxidised glutathione (GSSG; 10 mM) and NADPH (3 mM) (modified from Schaedle and Bassham[31]). GST activity was determined using a modification of the method of Habig and Jakoby.[32] The reaction of ethanolic 1-chloro-2,4-dinitrobenzene (CDNB; 10 mM) with potassium phosphate buffer (10 mM; pH 6·5) containing DTPA (0·2 mM) was monitored at 340 nm. Activity was calculated using 9·6 mM^{-1} cm^{-1} as the extinction coefficient of the conjugate.

CAT was analysed using potassium phosphate buffer (100 mM; pH 7·0) and hydrogen peroxide (0·5 ml litre^{-1}). The assay was based on the decrease in absorbance at 240 nm due to the reduction of hydrogen peroxide by CAT and activity was calculated as ΔA_{240}

mg^{-1} protein min^{-1}. GPX was assayed according to Horsman and Wellburn.[33] This was based on the reduction of hydrogen peroxide and the oxidation of guaiacol, an artificial electron donor, by GPX. Potassium phosphate buffer (100 mM, pH 6·0) containing DTPA (0·2 mM), hydrogen peroxide (0·5 ml litre ^{1}) and guaiacol (100 mM) was assayed for the change in absorbance at 470 nm and activity was calculated using an extinction coefficient for guaiacol of 26·6 mM ^{1} cm^{-1} at 470 nm.

2.7 Statistical analysis

All experiments consisted of two replicates of four treatments, namely control, ozone alone, phenmedipham alone, and ozone followed by phenmedipham. For each treatment four pots, each containing two plants, were used. In enzyme experiments this was reduced to two pots per treatment per replicate due to practical constraints. Pots were fully randomised within the growth cabinet. Growth and photosynthesis studies were performed four times, whilst enzyme experiments were repeated twice. Statistical analysis of the experiments, except enzyme assays, was conducted using Duncan's Multiple Range Test. Different letters in Table 1 indicate significant differences at the 5% level.

3 RESULTS

3.1 Symptom development

Leaves of plants treated with ozone developed chlorotic lesions (20% of leaf area), 1–2 mm in length, two or three days after exposure, whilst phenmedipham characteristically induced round chlorotic spots of 5–10 mm diameter (15% of leaf area), two to four days after spraying. Both phenmedipham and ozone injury occurred only on leaves that were present at the time of treatment. To quantify this response, shoot fresh weights, total chlorophyll and total carotenoid contents were determined. All treatments significantly reduced shoot fresh weight (Duncan's Multiple Range test, $P < 0{\cdot}05$; Table 1). Ozone exposure resulted in a 13% decline, whilst phenmedipham decreased fresh weights by 30%. Preliminary experiments were conducted to determine the response of sugarbeet to a range of phenmedipham concentrations. The recommended field rate was found to produce reductions in weight comparable to those found in the literature.[6] A 30% decline in fresh weight was observed in response to treatment with ozone followed by phenmedipham. Chlorophyll content was unaffected by ozone (Table 1) but clearly reduced following treatment with phenmedipham. When treated with both ozone and phenmedipham chlorophyll contents were intermediate between those treated with either ozone alone or phenmedipham alone. These results were mirrored by the total carotenoid contents, with similar responses to all three treatments.

3.2 Gaseous exchange

The rates of photosynthesis and transpiration were unaffected by a short-term exposure of ozone (Figs 1 and 2). However, plants treated with phenmedipham, or ozone followed by phenmedipham, showed a rapid decrease (56 and 58% respectively) in photosynthetic rate one day after spraying. A slight subsequent recovery was noted, but rates had not returned to control values six days after spraying. Similarly, transpiration rates were decreased by 25 and 31% for plants treated with phenmedipham alone and ozone and phenmedipham, respectively. Effects on transpiration were less dramatic than on photosynthesis.

3.3 Enzymes

The specific activities of the active oxygen scavenging enzymes were determined for the days following ozone exposure and herbicide treatment. The data for three critical days of the experiment, i.e. one day after ozone

TABLE 1
Effects of Ozone and/or Phenmedipham on Shoot Fresh Weight, Total Chlorophyll and Total Carotenoid Content of Sugarbeet cv. Saxon Seven Days after Herbicide Treatment[a]

Treatment	*Shoot fresh weight* (*g*)	*Total chlorophyll* (*μg g^{-1} fresh weight*)	*Total carotenoids* (*μg g^{-1} fresh weight*)
Control	1.610a	839.6a	185.7a
Ozone	1.407b	833.1a	189.2a
Phenmedipham	1.122c	466.0b	107.0b
Observed ozone and phenmedipham	1.130c	613.0c	137.5c
Expected ozone and phenmedipham	0.981	462.4	109.0

[a] Values are means, where $n = 4$–8. Expected values were calculated as the percentage reduction due to the herbicide subtracted from the value for ozone-treated plants. Different letters indicate significant differences at the 5% level according to Duncan's Multiple Range Test.

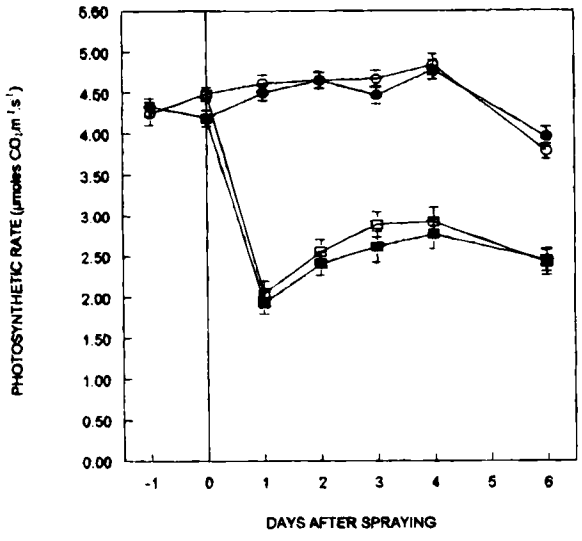

Fig. 1. The effects of ozone and/or phenmedipham on the photosynthetic rate of the first two leaves of sugarbeet cv. Saxon. (○) Controls, (●) ozone, (□) phenmedipham and (■) ozone and phenmedipham. Values are means ± SE, where $n = 4$.

exposure, immediately prior to spraying and three days after spraying, are shown in Table 2. A marked decline in the protein contents of plants treated with ozone alone and ozone and phenmedipham was observed (Table 2). Phenmedipham treatment produced a gradual decline in protein content to 75% of that of the untreated plants. There was no alteration in MDHAR activity in response to any treatment (data not shown).

The immediate effects of ozone (one day after exposure) were observed as increased APX, GTR and GST activities. The scavenging enzymes may be characterised according to their intracellular location: mainly

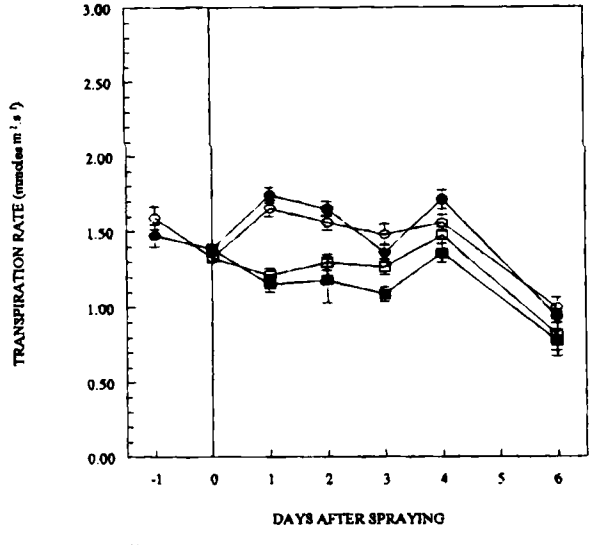

Fig. 2. The effect of ozone and/or phenmedipham on the transpiration rate of the first two leaves of sugarbeet cv. Saxon. (○) Controls, (●) ozone, (□) phenmedipham and (■) ozone and phenmedipham. Values are means ± SE, where $n = 4$.

chloroplastic (SOD, APX, GTR) and mainly cytosolic (GPX, CAT, GST). Plants treated with ozone showed higher activities of the enzymes which are mainly cytosolic in comparison to the controls (GPX 175% increase; CAT 40% increase and GST 70% increase) and smaller responses in those which are mainly chloroplastic (APX 25% increase and GTR 40% increase; Figs 3(a)–(f)), compared to untreated control plants. SOD, which has two isozymes, one of which is chloroplastic, the other mitochondrial, showed a negligible response to ozone. Phenmedipham treatment produced responses similar to those after ozone exposure, for SOD (20% increase), CAT (40% increase), GTR (45% increase) and GPX (180% increase) activities (Figs 3(a)–(f)), whilst a greater increase was detected in APX activity (50% increase). The activity of the glutathione conjugation enzyme, GST, was lower (140% of the control) in sugarbeet treated with phenmedipham than in those exposed to ozone alone (170% of the control).

When plants were exposed to ozone and then treated with phenmedipham, activities of SOD and APX were considerably higher than expected (85% and 125% increases respectively, Figs 3(a) and (c)), indicating an increased choroplastic response. A similar greater-than-additive response was observed in CAT activity (190% increase; Fig. 3(d)); however the remaining enzymes indicated activities which were additive in nature.

4 DISCUSSION

Phenmedipham induced chlorotic lesions similar to those described elsewhere.[2,6,7] These are thought to be due to contact action of the spray with the leaf surface, since they only appeared on leaves present at the time of spraying. Seven days after phenmedipham treatment, the fresh weight and chlorophyll content had decreased by 30% and 44% respectively. In an earlier study, phenmedipham decreased plant dry weight by 36–73%, with the magnitude of decrease depending on weather conditions at the time of spraying.[6] Symptoms of ozone injury observed in this study were typical of those reported for *Beta vulgaris* (garden beet).[34] In the present study, fresh weight of plants exposed to ozone was decreased by 13%, whereas the chlorophyll content remained unchanged. Previously, a 50% decrease in the shoot weight of garden beet was detected, although a higher concentration and dose of ozone were used (200 nl litre^{-1}, 1–3 h day^{-1} for five weeks).[34]

The response of plants treated with ozone followed by phenmedipham was not additive since the reduction in fresh weight was 30% and not the expected 39·1% (Table 1). Therefore, the combined effect of ozone and phenmedipham was similar to that of phenmedipham alone, indicating that a slightly less than additive interaction had probably occurred. Thus, it would appear that either ozone or phenmedipham, or both, were not

TABLE 2
Effects of Ozone and/or Phenmedipham on the Leaf Protein Content and Antioxidant Enzyme Activity[a]

	One day after ozone exposure		*Immediately before spraying*		*Three days after spraying (six days after ozone exposure)*			
	Control	*Ozone*	*Control*	*Ozone*	*Control*	*Ozone*	*Phenmedipham alone*	*Ozone + phenmedipham*
Protein	121·8	108·6	120·1	84·6	123·7	101·0	117·5	100·7
(mg per leaf)	(4·4)	(6·6)	(7·3)	(4·8)	(13·8)	(7·49)	(11·5)	(16·4)
APX	423·7	609·4	1052·7	1297·1	637·2	864·0	812·1	779·5
(nmol mg^{-1} protein min^{-1})	(26·7)	(55·5)	(41·2)	(81·0)	(75·2)	(58·5)	(67·1)	(76·4)
SOD	3·72	4·03	4·73	5·53	5·02	5·89	5·30	6·42
(units SOD mg^{-1} protein min^{-1})	(0·19)	(0·18)	(0·36)	(0·49)	(0·46)	(1·03)	(0·47)	(0·71)
GTR	49·8	71·2	47·8	67·1	49·9	60·4	66·2	77·7
(nmol mg^{-1} protein min^{-1})	(1·4)	(7·8)	(1·0)	(7·4)	(2·7)	(5·1)	(4·0)	(15·6)
GST	26·53	38·28	19·55	33·53	16·31	21·63	23·59	26·44
(nmol mg^{-1} protein min^{-1})	(2·43)	(4·55)	(2·85)	(4·50)	(0·76)	(2·07)	(1·21)	(3·51)
GPX	32·8	37·5	37·1	101·6	33·6	64·9	95·0	131·9
(nmol mg^{-1} protein min^{-1})	(4·8)	(4·1)	(3·4)	(27·1)	(7·3)	(10·3)	(7·0)	(18·3)
CAT	13·27	13·36	9·11	9·27	6·96	7·37	10·13	12·24
(ΔA_{240} mg^{-1} protein min^{-1})	(1·79)	(0·69)	(0·93)	(0·08)	(1·63)	(0·76)	(0·61)	(1·69)

[a] Values are means (±SE), where $n = 8$.

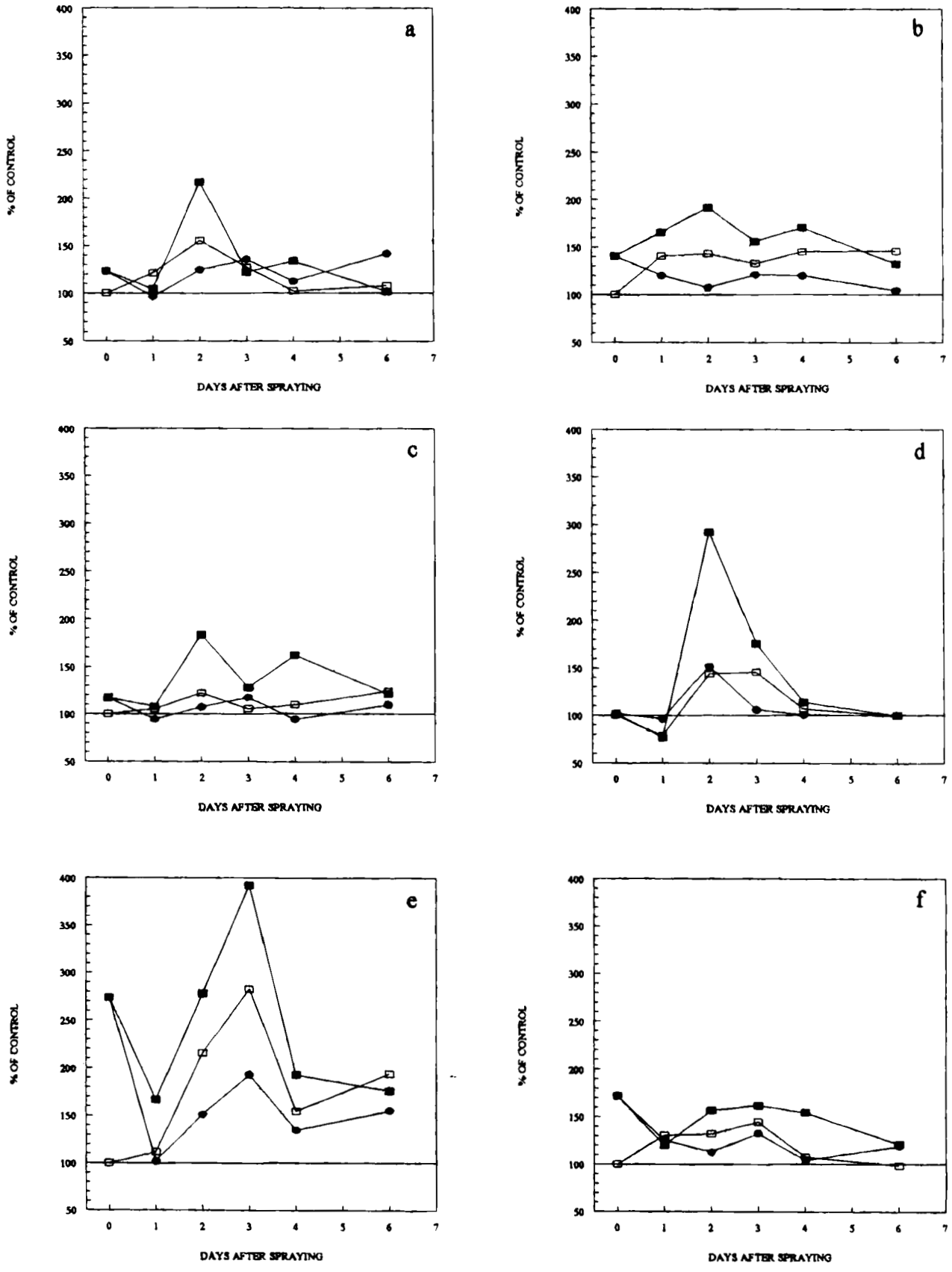

Fig. 3(a)–(f). The effects of (●) ozone, (□) phenmedipham and (■) ozone and phenmedipham on (a) ascorbate peroxidase, (b) glutathione reductase, (c) superoxide dismutase, (d) catalase, (e) guaiacol peroxidase and (f) glutathione-*S*-transferase in sugarbeet cv. Saxon. Values are means, where $n = 8$.

exerting their full effect when plants were exposed to both agents. Additional work was subsequently performed to establish the cellular location of the interaction.

This study initially focused on photosynthesis, as both ozone and phenmedipham have been reported to decrease carbon dioxide uptake in susceptible plants.[35,36] However, photosynthetic rate did not decrease in response to a simulated ozone episode, confirming the low susceptibility of sugarbeet to the concentration of ozone used. In contrast, phenmedipham decreased net photosynthetic rate by over 50% and rates had not recovered seven days after spraying. This decrease was greater than that observed previously, where carbon dioxide uptake in sugarbeet was reduced by 16%.[35] Plants treated with ozone followed by phenmedipham showed an expected decrease in photosynthetic rate similar to that of phenmedipham-treated plants (Fig. 1).

Concurrent measurement of rates of transpiration showed no effect of ozone exposure. Thus, stomatal conductance was maintained and presumed not to play a role in protecting sugarbeet against ozone pollution. Previous findings have shown that some ozone-tolerant cultivars of *Phaseolus vulgaris* L. have lower stomatal conductance than ozone-sensitive ones.[18,36] Phenmedipham induced some stomatal closure, since transpiration rates of plants decreased by 30% after spraying. Transpiration rates of plants treated with ozone followed by phenmedipham were similar to those treated with phenmedipham alone. As with photosynthesis, this was not unexpected, since ozone had no effect on transpiration rate. Thus, it seems unlikely that an inhibition of photosynthesis or changes in transpiration rates account for the observed interaction between ozone and phenmedipham.

Since ozone and herbicide tolerance have been linked with changes in antioxidant defence systems,[17,36] an investigation of the activity of key antioxidants with time was undertaken following treatment with ozone and/or phenmedipham. Ozone has a primary effect on the plasma membrane[37] and therefore would be expected to increase the activity of extracellular and cytoplasmic scavengers rather than chloroplastic antioxidants. There was an immediate increase in total cellular GST activity in sugarbeet, in response to ozone exposure (Table 2, Fig. 3(f)), supporting the results of the study in which *Hordeum vulgare* L. was exposed to a five-day ozone episode (200 nl litre^{-1}, 7 h day^{-1}).[38] In a similar study, a 26-fold increase in GST mRNA in *Arabidopsis thaliana* (L.) Heyhn. was observed 3 h after ozone exposure.[39] Clearly, activation of GST is an important response to ozone where it may function by catalysing the detoxification of lipid peroxides, conjugating glutathione with hydrophobic electrophiles. GST may also act as a peroxidase against free fatty acyl hydroperoxides.[38] Peroxidase enzymes in the intercellular space and those bound to cell walls are usually assayed using the non-specific electron donor guaiacol (GPX). In this study, induction of GPX activity by ozone was slower than that of GST, but was still an important ozone response, reaching a peak two or three days after exposure. Peroxidases are involved in cell-wall hardening, which could allow plants exposed to ozone to reduce their membrane permeability.[40] Catalase was used as an additional extra-chloroplastic marker, but showed no response to ozone (Table 2, Fig. 3(d)). Published findings on the response of CAT to ozone show a high variability that is dependent on the ozone concentration.[39,40] Consequently the role of CAT is not clear.

As a large proportion of total cellular SOD is located in the chloroplast,[41] this enzyme was used as a chloroplastic marker. There was no response of SOD to ozone, which was expected if the primary site of ozone damage was outside the chloroplast. However, previous studies have indicated that the response of SOD to ozone is highly variable and depends on species susceptibility[42] and the ozone concentration.[43] Enzymes within the ascorbate-glutathione regeneration system are found within distinct cellular compartments, but several isozymes of some enzymes exist. For example, cytoplasmic and chloroplastic isozymes have been identified for both APX and GTR.[44,45] Since total cellular activities were measured in this study, it was not possible to identify which isozymes of APX or GTR increased activity in response to ozone (Table 2). However, given that other chloroplastic processes were not affected by ozone, it is unlikely that increases in activity were due to chloroplastic isozymes.

The primary site of phenmedipham damage is in the chloroplast, where the herbicide blocks photosynthetic electron transport.[5] This was manifest as a 50% reduction in photosynthetic rate (Fig. 1). Excitation energy is not dissipated by PSI and PSII, leading to the generation of triplet-state chlorophyll which sensitises the formation of singlet oxygen and lipid peroxidation. This ultimately leads to destruction of the thylakoid membranes[17] and explains the large decreases in chlorophyll and carotenoid contents shown in Table 1. SOD activity also increased, confirming a mainly chloroplastic response. As known chloroplast markers were affected by phenmedipham, the increases in APX and GTR detected in plants treated with phenmedipham were more likely to be due to chloroplastic isozymes. However, there were increases in the activities of both GPX and CAT, which would indicate a secondary extrachloroplastic response. Phenmedipham had a smaller effect on GST activity than did ozone exposure. It is not known whether phenmedipham is conjugated with glutathione, although the thiocarbamate EPTC is metabolised in this manner.[46] This effect of phenmedipham on GST activity may have been due to the peroxidase activity of the enzyme against free fatty acyl

hydroperoxides produced as a result of the herbicide's action on the cellular membranes.

These observations are consistent with the hypothesis that initial treatment with ozone activated the antioxidant scavenging system, so that when the plants were subjected to an additional oxidative stress by phenmedipham, antioxidant status was already elevated. Total cellular SOD was greater than additive, indicating a chloroplastic response to treatment with ozone followed by phenmedipham. Plants exposed to both treatments also showed greater chlorophyll and carotenoid contents compared to those treated with phenmedipham alone. This implied that less damage was occurring in the chloroplast, although photosynthetic rate had decreased by a similar amount. Hence, the greater-than-additive response of APX observed in this study may also be attributable to the chloroplastic isozyme. It is not clear why ozone and phenmedipham treatments together increased the activity of CAT by 190% (Fig. 3(d)). Plants did not show an additional increase in GST activity in response to phenmedipham treatment after ozone exposure, although activities declined more slowly than after ozone exposure.

The primary sites of damage of ozone and phenmedipham are different.[5,36] Phenmedipham acts primarily in the chloroplast, where it blocks electron transport at the thylakoid membrane leading to the production of active oxygen species, and, consequently, enzymes largely associated with the chloroplast are more active. Conversely, ozone damages the plasma membrane and activities of antioxidant enzymes in this vicinity are stimulated, for example, GPX, GST and isozymes of GTR. Induction of largely chloroplastic enzymes including APX and SOD is a secondary response to ozone in sugarbeet. When the herbicide is sprayed after ozone exposure then both the plasma membrane and the chloroplast are potential damage sites. From the findings presented in this study, it would appear that ozone stimulates the antioxidant system, so that, if an additional oxidative stress, such as a PSII herbicide, is imposed on the plant, it is more able to deal with the generation of active oxygen species. The net effect is the response seen in the reduction of fresh weight, since the herbicide does not exert the full predicted effect. This study supports the hypothesis that improving the endogenous antioxidant capacity of plants can lead to increased stress tolerance.[47]

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